APPLIED MICROBIOLOGY, Feb. 1975, p. 219-223

Vol. 29, No. 2
Printed in U.S.A.

Production and Purification of the Thermophilic Bacteriophage TP-84

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Received for publication 7 August 1974

A new procedure for production and purification of the thermophilic bacteriophage TP-84 in high yields is described. Cultures of *Bacillus stearothermophilus* strain 10, enriched with nutrients to obtain heavy growth and to prevent sporulation and maintained at a pH of 6.5, were infected with the phage in a 100-liter fermentor. Addition of magnesium chloride (0.01 M) and a temperature of 58 C were essential for maximal phage production. Phage (5×10^{11} infective particles/ml) was precipitated with polyethylene glycol (molecular weight 6,000) in the presence of sodium chloride and was further purified by cesium chloride density centrifugation.

Since the first isolation by Koser (12, 13) of a thermophilic bacteriophage, many other thermophilic phages have been described. Like the mesophilic phages, the thermophilic ones can be subdivided into two main groups: (i) virulent thermophilic phages (2-7, 10, 12, 13, 16, 18, 19, 27, 28) and (ii) temperate phages (7, 17, 20-23, 25, 26). Unique in their extreme thermal stability (6, 10, 12, 13, 16, 19, 20, 25, 27, 28), the thermophilic phages have attracted the attention of many investigators. Our interest in the thermophilic phage TP-84 derives from the assumption that thermophilic phages may represent the simplest form of thermophilic organisms and thus the mechanism(s) for their remarkable thermostability might be easier to elucidate. This report deals with the mass production and purification of bacteriophage TP-84. Such work is a prerequisite for a detailed analysis of the structural components of the phage and the physical-chemical characterization of the phage proteins.

MATERIALS AND METHODS

Host organism. *Bacillus stearothermophilus* strain 10 (19) was used as the host organism.

Bacteriophage. The thermophilic bacteriophage used was isolated from greenhouse soil in 1952 by one of us (L. L. C.), using *B. stearothermophilus* strain 2184 as the host organism. Some of its properties have been reported (3, 18, 19).

Media and culture conditions. The media and growth conditions used in the early stages of this work have been described (19).

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The medium used for growth of the host organism and phage production (TYC medium) contained: Trypticase (BBL), 20 g; yeast extract, 3 g; NaCl, 8.5 g; $CaCl_2 \cdot 2H_2O$, 1.47 g; $FeCl_3 \cdot 6H_2O$, 7 mg; $MgSO_4 \cdot 7H_2O$, 15 mg; and $MnCl_2 \cdot H_2O$, 1 mg per liter of distilled water. The medium was adjusted to pH 7.4 (19). As the work progressed, it was found that certain components of the TYC medium destroyed the biological activity of phage TP-84 (to be reported elsewhere); thus a modified medium (TYM) was developed. TYM medium contains: Trypticase, 20 g; yeast extract, 4 g; $MgCl_2 \cdot 6H_2O$, 2 g; $CaCl_2 \cdot 2H_2O$, 0.73 g per liter of distilled water. The pH was adjusted to 6.5.

Bacteriophage TP-84 was propagated on its host organism, *B. stearothermophilus* strain 10. Unless otherwise specified, the conditions for growth of this strain and phage production were as follows. A logarithmic culture of the host was grown in TYM medium in a rotatory shaking water bath (New Brunswick Scientific Co., New Brunswick, N.J.) with vigorous aeration at 58 C. As the culture reached a turbidity of either 200 or 400 Klett units (KU), depending on the experiment, it was infected with purified TP-84 suspension at a multiplicity of infection of 0.01.

Phage assay. Phage TP-84 was assayed by the soft agar overlap technique (1). The base layer contained 25 ml of either 2% TYC or 2% TYM agar. The overlay medium contained 2.5 ml of either 0.6% TYC agar or 0.6% TYM agar, respectively, 0.3 ml of an overnight culture of *B. stearothermophilus* 10, and 0.1 ml of phage suspension. All phage dilutions were done in either TYC or TYM medium. The plates were incubated for 18 h at 55 C. The concentration of infective phage particles in a phage suspension is expressed as plaque-forming units (PFU) per milliliter.

Purification of TP-84. Initially we used the purification technique developed by Saunders and Campbell (19). However, during the $(NH_4)_2SO_4$ stage of the purification at least 50% of the phage was lost. To

overcome this problem, we used either the magnesium carbonate adsorption-solubilization technique (Fig. 1) or the NaCl-polyethylene glycol precipitation technique (29), described in Fig. 2. With either technique, the final purification of the phage was done by CsCl density gradient centrifugation as described by Saunders and Campbell (19). Solid CsCl (purity 99.9%, Penn Rare Metals Division) to a final density of 1.51 g/ml was added either directly to the dissolved magnesium carbonate-phage suspension or to the NaCl-polyethylene glycol-precipitated phage after it was suspended in TMC buffer containing 0.5 M tris(hydroxymethyl)aminomethane, 0.01 M $MgCl_2 \cdot 6H_2O$, and 0.005 M $CaCl_2 \cdot 2H_2O$, pH 7.3. The density of all fractions was determined by measurement of the refractive index (Abbe 3-L refractometer, Bausch & Lomb, Rochester, N.Y.; [24]).

Bacterial count. The number of viable cells of *B. stearothermophilus* present in a sample was determined by the standard viable count technique; 0.1 ml of the proper dilution was spread over TYM agar, the plates were incubated for 18 h at 55 C, and the colonies were counted.

Effect of NaCl and $MgCl_2 \cdot 6H_2O$ on phage production. Baffle-bottomed flasks adapted with a side-armed colorimetric tube were used. T medium (2% Trypticase and 0.4% yeast extract, pH 7.0) was supplemented with various concentrations of either NaCl or $MgCl_2 \cdot 6H_2O$. For comparison, the experiment was also done in TYM and TYC (19) media. Each of the 250-ml flasks with 100 ml of the appropriate medium was inoculated with a logarithmic culture of *B. stearothermophilus* strain 10 and incubated in a rotatory shaking water bath (120 strokes/min) at 58 C. The turbidity was measured with a Klett-Summerson photoelectric colorimeter with a no. 54 filter (540 nm). As the cultures reached a turbidity of 200 KU, phage TP-84 was added at a multiplicity of infection of 0.01. The time needed by the different cultures to lyse was recorded, and the number of PFU produced per milliliter was assayed.

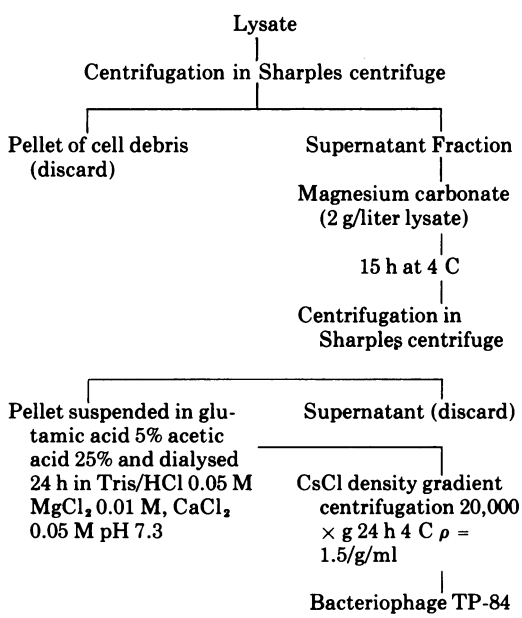

FIG. 1. *Purification of bacteriophage TP-84 by the magnesium carbonate adsorption-solubilization technique.*

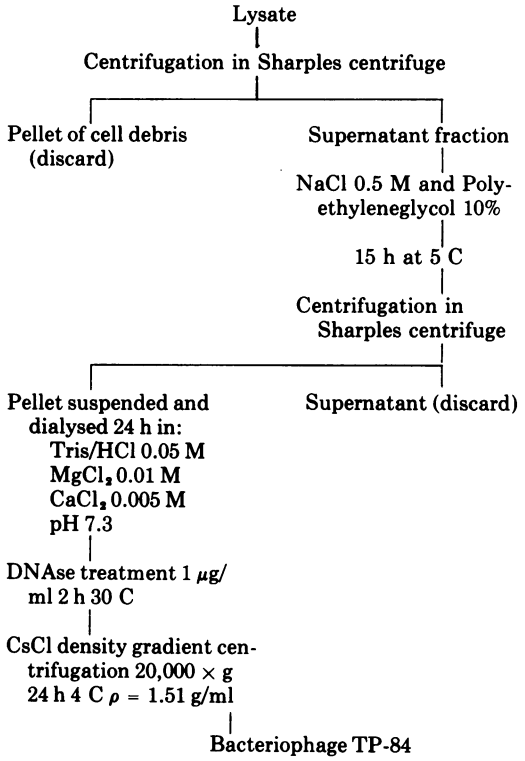

FIG. 2. *Purification of phage TP-84 by the polyethylene glycol-NaCl technique.*

Effect of pH on phage production. TYM medium was adjusted to different pH values in separate baffle-bottomed flasks (100 ml of medium per 250-ml flask), and each flask was inoculated with a culture of *B. stearothermophilus* strain 10 grown at the respective pH. The cultures were incubated with shaking (120 strokes/min) at 58 C. When the turbidity of the cultures reached 200 KU (540 nm), they were infected with TP-84 at a multiplicity of infection of 0.01. Throughout the experiment, the pH was maintained at its initial value by the addition of HCl (1 N) or KOH (1 N) by using a Corning digital 112 pH meter (Corning Scientific Instruments). The number of PFU produced per milliliter by the different cultures was assayed by the soft agar technique (1).

RESULTS

Effect of NaCl concentration on phage production. Saunders (Ph.D. thesis, Univ. of Illinois, Urbana, 1965) reported that the lytic cycle of phage TP-84 does not start unless NaCl

is added to the medium. He assumed that NaCl enables phage adsorption to its host. Similar findings were reported (15) for a filamentous bacteriophage. However, in the case of phage ΦX174 (9), divalent cations, and not NaCl, were important for adsorption. The effect of NaCl concentration (0, 0.05, 0.1, or 0.15 M) on phage TP-84 production was analyzed as described in Materials and Methods. The results revealed no NaCl dependency during phage production, including the stage of phage adsorption. Under the conditions tested the cultures lysed almost at the same time, and the final PFU produced per milliliter were comparable. At high NaCl concentrations (0.1 M) smaller phage titers were found. Therefore, NaCl is not essential for phage production.

The effect of Mg ion concentration on phage production. Effect of $MgCl_2$ on phage production was tested in T medium as described in Materials and Methods. It has been shown (3, 19) that chelating agents such as ethylenediaminetetraacetic acid and phosphate cause dissociation of the phage TP-84 into its head and tail and cause the release of deoxyribonucleic acid. We have shown (manuscript in preparation) that certain divalent cations such as magnesium and calcium increase the structural and thermal stability of phage TP-84. In the presence of 0.01 M $MgCl_2 \cdot 6H_2O$, 3×10^{10} PFU were produced per ml (Table 1), whereas in T medium (without magnesium) less than 1% of phage was produced (1.5×10^8 PFU/ml). No further increase in the phage titer was obtained by increasing the $MgCl_2$ to 0.1 M. As will be discussed later, magnesium ions primarily affect phage stability and not production.

The effect of pH of the medium and growth temperature on phage production. Maximal phage production depends upon the composition of the growth medium; however, the pH of the medium and growth temperature are also very important. The optimal growth temperature of *B. stearothermophilus* is 65 C, whereas maximal phage production was obtained at 58 C. The highest yield of phage was produced by the culture grown at pH 6.5 (Table 2). Only few phage particles were produced by cells grown at pH 5.5 and 8.5. A good correlation exists between the final number of phage particles produced at the different pH values and the time required by the infected cultures to lyse. In the pH 6.5 culture, the highest yield of phage was obtained and the lytic cycle was the shortest (about 45 min); lysis occurred at 50 min in the culture kept at pH 7.5, and the yield of phage was lower. Lysis was greatly delayed at pH 5.5 and 8.5.

TABLE 1. *Effect of magnesium chloride on phage TP-84 production*

Medium composition	MgCl added (M)	No. of phage particles produced per ml
T[a]	0	1.5×10^8
T	0.001	4.0×10^8
T	0.01	3.0×10^{10}
T	0.1	9.0×10^9

[a] T medium contained: Trypticase, 20 g, and yeast extract, 4 g per liter of distilled water. The pH was adjusted to 6.5. Cultures of *B. stearothermophilus* were grown in the medium specified in the table at 58 C up to a turbidity of 200 KU (λ = 540 nm) and infected with pure phage TP-84 at a multiplicity of infection of 0.1.

TABLE 2. *Effect of pH of the medium on phage TP-84 production*[a]

pH of the medium at infection	pH of the lysate	Phage particles produced per ml	Time required for completion of lysis (min)
5.5	5.5	6.0×10^8	180
6.0	6.2	5.0×10^{10}	80
6.5	6.8	6.0×10^{11}	45
7.0	7.1	7.0×10^{10}	43
7.5	7.6	4.0×10^{10}	50
8.0	8.0	3.2×10^9	120
8.5	8.5	2.0×10^7	240

[a] Cultures of *B. stearothermophilus* grown (58 C) in TYM broth at the desired pH were infected with pure phage TP-84 at a multiplicity of infection of 0.1. The pH was maintained at its original value by addition of either HCl (1 N) or KOH (1 N).

Correlation between the number of viable cells of B. stearothermophilus strain 10 at infection and final PFU produced per milliliter. A direct relationship between the number of TP-84 particles produced and the viable count of *B. stearothermophilus* at infection was expected. However, this was not the case. Only in cultures infected at turbidities of 250 KU or less was there a direct correlation between the viable cell count and the PFU produced per milliliter (Table 3). The final number of PFU per milliliter increased from 2.0×10^9-5.0×10^9 to 2.0×10^{10}-4.0×10^{10} PFU/ml, when the viable count at infection was 3.1×10^7 and 6.7×10^8 cells/ml, respectively. As the turbidity of the cultures increased and the cultures approached either the late log phase or the stationary phase of growth (300 KU and above), the yield of phage leveled off. At higher turbidities of 300 to 500 KU (viable counts of 8.0×10^8 and 1.9×10^9 cells/ml, respectively), the phage count re-

TABLE 3. *Correlation between phage TP-84 production, viable count of B. stearothermophilus, and fructose in the medium*[a]

Turbidity at infection (KU[λ = 540 nm])	Viable count (cells/ml)	Fructose added before infection	Time needed for lysis to occur (min)	Phage count (PFU/ml)
25	3.1×10^7		45–70	2.0×10^9–5.0×10^9
40	6.7×10^7		45–70	2.0×10^9–5.0×10^9
115	1.8×10^8		50–80	5.0×10^9–9.0×10^9
150	3.1×10^8		50–80	1.0×10^{10}–2.5×10^{10}
250	6.7×10^8		60–100	2.0×10^{10}–4.0×10^{10}
450	9.7×10^8		No lysis	
500	1.9×10^9		No lysis	
500	1.8×10^9	0.5% (final concentration)	40–70	1.2×10^{11}–5.0×10^{11}

[a] Cultures of *B. stearothermophilus* were grown in TYM medium (58 C) and infected with phage TP-84 (multiplicity of infection, 0.01) at various turbidities (λ = 540). A sample was taken from each culture before addition of phage for viable count of the host. The time required by each culture to lyse was recorded, and the number of phage particles produced was determined.

mained almost the same as that obtained at 250 KU. Frequently, the high-density cultures did not lyse even 24 h after infection. Such cultures consisted predominantly (up to 90%) of refractile spores, as judged by phase contrast microscopy. Though the cultures had not lysed, phage was present. When cells were washed to remove phage and the washed cells were added to a logarithmically growing culture of *B. stearothermophilus*, a new lytic cycle began. Thus, the phage were attached to or were inside the cells committed to sporulation but further phage production was arrested (see reference 11).

To postpone or prevent the onset of sporulation, Trypticase and yeast extract at final concentrations of 2% and 0.4%, respectively, were added when the turbidity of the cultures reached 300 KU (540 nm; see reference 14). Thirty minutes later (the turbidity of the cultures was approximately 400 KU), fructose (see reference 8) at a final concentration of 0.5% was added to one culture, and none was added to the control. A purified phage suspension was added to both cultures at a multiplicity of infection of 0.01. Approximately 45 to 60 min later, lysis occurred only in the culture to which fructose was added. The latter conditions resulted in a 10-fold increase in phage production from an average maximum of 2×10^{10} to 5×10^{10} PFU/ml in TYM medium to 2×10^{11} to 5×10^{11} PFU/ml by the improved method described.

Purification of TP-84. Saunders and Campbell (19) used ammonium sulfate precipitation for concentrating the phage TP-84 from mass lysates. However, frequently 50 to 70% of the phage was lost during the treatment. Therefore, a more efficient purification procedure was developed. The scheme outlined in Fig. 1 describes the purification of phage TP-84 with magnesium carbonate. The lysate was stirred overnight at 4 C with magnesium carbonate. After centrifugation, the slurry of magnesium carbonate with the adsorbed phage was dissolved by the slow addition of a mixture of glutamic and acetic acids (25% acetic acid and 5% glutamic acid). The final purification step was done by CsCl density gradient centrifugation as described earlier (19). Later, a combination of the method of Yamamoto and Alberts (29) and CsCl density gradient centrifugation (Fig. 2) was used. We found the latter technique easier, less expensive, and more efficient. Whereas 80% of the phage was recovered by the magnesium carbonate method, the recovery by the NaCl-polyethylene glycol technique was at least 90%.

DISCUSSION

Thermophilic bacteriophages are the smallest thermophilic organisms known. A phage particle is a heteropolymeric complex of molecules composed of structural proteins and genetic material. Two distinct biological activities can be detected in the free phage particle, infectivity of the phage and contractility of the tail structure. In phage TP-84 these activities are stable up to 75 C. Thus, a simple system for the analysis of the mechanism of thermophily is at hand. It is true that infectivity is lost soon after the phage particle is dissociated into heads and tails. Nevertheless, other methods are available to analyze the effect of temperature upon the various types of structural proteins of the phage. However, such a study cannot be carried out without efficient techniques for mass production and purification of the phage. Assuming that the final number of phage particles produced in a culture is dependent upon the burst size times the number of infected cells, we tried to infect cultures of higher turbidities. However, such dense cultures yielded very little phage. Thompson and Shafia (23) have shown that irreversible adsorption of the thermophilic phage ΦM-4 requires functional terminal respiration. Using thermophilic phage TP-84, we demonstrated that commitment of the host to

spore formation prevented further phage production. Unless sporulation was prevented and the cultures were maintained in the logarithmic phage, phage production was hindered. Addition of nutrients such as Trypticase, yeast extract, and fructose to the cultures prior to infection delayed sporulation and resulted in higher phage production with titers as high as 2×10^{11} to 5×10^{11} PFU/ml. Together with the very efficient purification technique described here, approximately 2×10^{16} PFU of phage TP-84 were purified from 100 liters of lysate. Quantities of this order are essential for purification of the phage proteins and determination of their primary structure.

ACKNOWLEDGMENTS

We gratefully acknowledge the interest and the advice of M. Grossowicz in preparation of this manuscript.

This investigation was supported by Public Health Service research grant AI-06885 from the National Institute of Allergy and Infectious Diseases.

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